Compartmentalization of the Inflammatory Response to Inhaled Grain Dust

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Interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α, and the secreted form of the IL-1 receptor antagonist (sIL-1RA) are involved in the inflammatory response to inhaled grain dust. Previously, we found considerable production of these cytokines in the lower respiratory tract of workers exposed by inhalation to aqueous extracts of corn dust extract. Alveolar macrophages (AM) have long been considered the cell type responsible for producing these cytokines, and only recently has it been realized that airway epithelial cells may also be involved in cytokine production. In order to determine whether airway epithelia are involved in the inflammatory response to inhaled corn dust extract and to compare the magnitude of response of bronchial epithelial cells (BE) and bronchoalveolar lavage (BAL) cells, we used the reverse transcriptase/polymerase chain reaction (RT/PCR) technique in a semiquantitative manner to evaluate the concentration of IL-1β, IL-6, IL-8, TNF-α, and sIL-1RA. Alveolar cells were obtained by BAL, and BE were obtained by endobronchial brush biopsy from 15 grain handlers 6 h after experimental inhalation of saline or an aqueous corn dust extract. After inhalation of saline, BE expressed low but detectable levels of IL-6, IL-8, and IL-1β (> 1 complementary DNA [cDNA] molecule/cell). After inhalation of corn dust extract, the expression of messenger RNA (mRNA) for IL-1β and IL-8 in the BE were significantly increased, whereas no change was seen in IL-6, sIL-1RA, and TNF-α mRNA expression. Comparing cytokine mRNA levels in BE and BAL cells from the same subjects after inhalation of corn dust extract, BE and BAL cells expressed equivalent amounts of IL-8 mRNA; IL-1β was 11-fold higher in BAL cells; and TNF-α and sIL-1RA were expressed exclusively by BAL cells. Immunostaining for the cytokines in BAL cells showed cytokine protein expression in AMs but not in polymorphonuclear cells (PMNs). On the other hand, sIL-1RA was strongly expressed in both AMs and PMNs. Analysis of cytokine protein levels in endobronchial lavage (EBL) fluid demonstrated that only IL-8 was released in detectable amounts into the airway lumen, whereas all the other cytokines of interest were exclusively found in the BAL fluid. Thus, within 6 h after inhalation exposure to corn dust extract, BE appear to contribute to airway inflammation by producing IL-8. AMs are responsible for most of the IL-1β and IL-6 production in the alveolar region, whereas AMs and PMNs both produce sIL-1RA. Our findings suggest that the inflammatory response to inhaled grain dust is compartmentalized, involving specific mediators of inflammation released by macrophages, neutrophils, and airway epithelial cells. Becker S, Clapp WA, Quay J, Frees KL, Koren HS, Schwartz DA. Compartmentalization of the inflammatory response to inhaled grain dust. AM J RESPIR CRIT CARE MED 1999;160:1309–1318.

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Inhalation of grain dust is associated with the development of airflow obstruction, polymorphonuclear cell (PMN) recruitment to the lung, and an increase in the concentration of PMNs in the bloodstream (1–3). Recently, we found that several proinflammatory cytokines were induced and recovered in bronchoalveolar lavage (BAL) fluid 6 h after inhalation exposure of corn dust extract (3). Analysis of cells obtained from the BAL fluid revealed significant increases in messenger RNA (mRNA) for cytokines interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α. In addition, the BAL fluid concentration of soluble interleukin-1 receptor antagonist (sIL-1RA) was induced by the inhalation challenge, and the concentration of sIL-1RA was > 100-fold that of IL-1β. However, the mechanisms underlying the development of grain-dust-induced airflow obstruction are poorly understood.

Organic dusts are frequently contaminated by endotoxin and the inflammatory cell and cytokine response induced by these dusts may be in large part or solely caused by this microbial product (4). In studies conducted with animals or with human populations exposed experimentally or occupationally to cotton dust, endotoxin has been found to be the primary cause for changes in airway reactivity and inflammation (5, 6). Atopic and nonatopic grain handlers, as well as workers not occupationally exposed to grain dust, appear to have a similar acute physiologic and biologic response to inhalation of grain dust, indicating that prior sensitization is not required to respond biologically or physiologically to grain dust (3). Moreover, the development of airflow obstruction after challenge with organic dust is not dependent on asthma or underlying airway hyperreactivity (3, 5).

Although alveolar macrophages (AM) are known to be important producers of a number of polypeptide mediators during inflammatory events in the lung, the epithelial cells may also be involved in recruitment and modulation of the inflammatory response. Previously, we found that IL-8 was expressed in isolated, uncultured, normal nasal and bronchial epithelium (7, 8). Low amounts of IL-1β and IL-6 mRNA were also found in bronchial epithelial cells (BE), suggesting that these cytokines could be produced upon stimulation of the airways. Experiments with airway epithelial cell lines and primary epithelial cell cultures have found that IL-6, IL-8, and granulocyte macrophage colony-stimulating factor (GM-CSF) are produced by these cells and can be regulated by TNF-α and IL-1β (9, 10) as well as by virus infection (11) and ozone exposure (12). Furthermore, these three cytokines (IL-6, IL-8, and GM-CSF appear to be expressed at elevated levels in asthmatic airway cells and are produced *in vitro* in excess amounts by epithelial cells from allergic persons (13, 14). On the other hand, compared with alveolar macrophages, airway epithelial cells require a 1,000-fold higher concentration to endotoxin to produce mRNA for IL-8 (15).

Thus, in order to determine the biologic events leading to grain-dust-induced airflow obstruction, it is important to understand the role of structural and inflammatory cells in mediating and localizing the inflammatory response to inhaled grain dust. In the present study, bronchoalveolar lavage, endobronchial brush biopsy, and endobronchial lavage were performed on 15 subjects after inhalation of saline and then, approximately 14 d later, after inhalation of corn dust extract. Cytokine mRNA content in the epithelial cells was determined and compared with the levels in cells obtained by BAL from the same subjects. Furthermore, the concentration of cytokines in endobronchial lavage fluid from the central airways was compared with the concentration of cytokines in the BAL. The results suggest an important role of epithelial cells in PMN recruitment to the airways through the production of IL-8. Importantly, AMs contribute to the inflammatory response through the production of TNF-α, IL-1β, and sIL-1RA.

METHODS

Subjects

The study population consisted of 15 men, selected randomly from approximately 200 grain handlers who are participating in a population-based longitudinal study of grain-dust-induced lung disease (3). The subjects were required to be nonatopic, have normal baseline lung function, and have a negative histamine bronchoprovocation test. They were also required to be nonsmokers for at least 2 yr prior to this study, have no underlying medical illness, and receiving no current medications. Each subject was also required to have an unremarkable chest radiograph and electrocardiogram. The institutional review board approved this study, and all study subjects signed an informed consent form.

Preparation of Inhaled Solution

Grain dust was obtained from a collection receptacle of an air filtration system at a corn storage facility. The dust had accumulated during the 2 wk prior to collection. The dust was placed in plastic containers that were sealed and stored at 9° C. Extracts were produced by mixing 3 g of the dust with 30 ml buffered saline (Hanks' BSS; Media Tech, Inc., Herndon, VA) followed by shaking for 60 min, centrifugation at 3,000 rpm, and then filter sterilization through a 0.45-μm filter (Acrocap; Gelman Sciences, Ann Arbor, MI). The endotoxin concentration of the extract solution was approximately 7 μg/ml as determined by chromogenic *Limulus* amoebocyte lysate assay (QCL-1000; Whittaker Bioproducts, Walkersville, MD). The pH of the control buffered saline was adjusted to 5.3, which was the measured pH of the corn dust extract. The solutions were stored in 15-ml aliquots at −70° C prior to use.

Protocol

All subjects were admitted to the Clinical Research Center at the University of Iowa Hospitals and Clinics the night before the inhalation challenge, and a standard protocol was followed in all cases. A screening evaluation, which included a complete history and physical examination, pulmonary function tests (spirometry, lung volumes, and diffusing capacity of carbon monoxide [$D_{L_{CO}}$]), a chest radiograph, and an electrocardiogram were performed on the evening of admission. At 6:30 the next morning, a plastic intravenous catheter was placed in the right forearm (heparin lock) and a peripheral blood sample was obtained. At 7:00 A.M., baseline forced expiratory maneuvers were performed using a Spirotech S-500 spirometer (Graseby Anderson, Atlanta, GA) with standard protocols following American Thoracic Society guidelines (16). Subjects were then exposed to nebulized buffered saline (first visit) or corn dust extract (second visit, at least 14 d after the first visit) by inhalation challenge. The aerosol challenge lasted approximately 60 min. Vital signs and forced expiratory maneuvers were measured at 30 min, 1 h, and hourly after the inhalation challenge was completed. Peripheral venous blood was obtained 5 h after inhalation challenge, and 1 h later, bronchoscopy was performed. Blood samples were processed for leukocyte and differential cell counts by our hospital clinical laboratories using standard protocols.

Inhalation Challenge

Subjects were exposed to nebulized buffered saline (first visit) and corn dust extract (second visit; at least 14 d after the first visit) by inhalation challenge. The solutions were administered via a DeVilbiss 646 nebulizer and DeVilbiss dosimeter (DeVilbiss Health Care Inc., Somerset, PA), operated at an air pressure of 20 psi. The subjects controlled the timing of each nebulized dose by arming the dosimeter before inhalation. The dosimeter automatically discharged for 0.6 s when triggered by the pressure drop in the nebulizer from inhalation. The port cap of the nebulizer was closed and the subject exhaled through his nose. The dose delivered was measured by changes in weight of the nebulizer. The mean extract delivered was 0.08 ml/kg. This resulted in delivery of between 4.5 and 8.1 ml to each subject, corresponding to between 30 and 60 mg of endotoxin, a dose that could be inhaled by an agricultural worker in a dusty environment over the period of an 8-h work shift (17).

Bronchoscopy

Bronchoscopy was performed in accordance with the standards established by the American Thoracic Society for bronchoscopy of asthmatics (18). After premedication with 50 to 100 mg meperdine and 0.6 mg atropine intramuscularly, 4% lidocaine was aerosolized to topically anesthetize the larynx. An Olympus 1T-10 fiberoptic bronchoscope (2.0 mm channel) was introduced through the oral cavity and the bronchoscope was gently passed into the trachea.

For BAL, an Olympus P-10 (1.5 mm channel) bronchoscope was introduced into the right middle lobe and wedged in the medial segment, where 20 ml of sterile 37° C saline were introduced. Immediately afterward, suction was gently applied (60 mm Hg), and the effluent was collected in a 50-ml specimen trap (Cheesebrough-Ponds Inc., Greenwich, CT). This was repeated five more times for a total volume of 120 ml. At the second visit, the BAL was performed in a subsegment of the lingula.

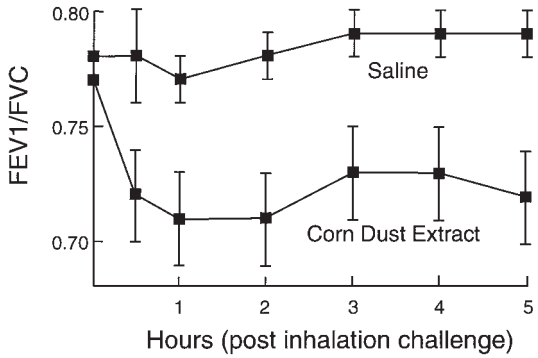

Figure 1. Percentage of baseline FEV_1/FVC after inhalation challenges with buffered saline or corn dust extract (± SEM).

The endobronchial lavage was performed by using a modified double-balloon pulmonary artery catheter (19). The catheter was introduced through the bronchoscope and passed into the left main-stem bronchus. The balloons were inflated with 2 to 3 ml air to occlude the proximal and distal portions of the bronchus, and the resulting seal was tested with gentle suction. Then eight 1.5-ml aliquots of 37° C saline were introduced through the catheter into the resulting space. Each aliquot was allowed to dwell for 30 s, then withdrawn. This was repeated until 12 ml were introduced, after which the catheter was withdrawn. Unfortunately, in seven study subjects, this procedure resulted in uncontrollable cough, and, thus, paired collection of endobronchial lavage fluid after both inhalation of saline and corn dust extract was accomplished in only eight study subjects.

After the endobronchial lavage (EBL), a no. 4 sheathed biopsy brush (Microvasive Microbiology Specimen Brush; Microvasive Microbiology, Watertown, MA) was introduced through the bronchoscope, and the right side of the trachea was brushed vigorously. The brush was resheathed and withdrawn. At the second visit, the biopsy was performed on the left side of the trachea. After removal from the bronchoscope, the brush was unsheathed and, using sterile technique, it was cut off into a sterile 1.5-ml centrifuge tube containing 1 ml of RPMI 1640 medium. The solution was agitated, the brush was removed, and the cells were counted in a hemacytometer. The cells were pelleted and 10^6 cells were dissolved in 1 ml 4 M guanidine isothiocyanate (GITC) for preparation of RNA by a miniprep method (20). Differential counts were performed on cytocentrifuge preparations stained with Diff-Quik (Harleco, Gibbstown, NY).

Preparation of Lavage Fluid and Cells

The EBL and the BAL fluids were processed in a similar manner. Immediately after bronchoscopy, the fluids were strained through two layers of surgical 4 × 4 gauze into 50-ml conical tubes. The volumes were noted and the tubes were centrifuged for 5 min at 200 × *g*. The supernatant fluids were frozen at −70° C for subsequent cytokine determination. The cell pellets were resuspended and washed twice in Ca^{2+}- and Mg^{2+}-free Hanks' BSS. After the second wash, small aliquots of the samples were taken for cell count using a hemacytometer, and cytocentrifuge preparations were done for differential counting. One million BAL cells were resuspended in 1 ml 4 M GITC for mRNA preparation (20). To create relatively pure cell populations for immunohistochemistry, after inhalation of corn dust extract, BAL fluid was separated over a 40% Percoll gradient (Sigma Chemical, St. Louis, MO), 500 × *g*, 20 min, into > 95% pure AM (interphase) and PMN (pellet).

Reverse Transcriptase/Polymerase Chain Reaction

Complementary DNA (cDNA) was generated from purified RNA corresponding to approximately 10^4 epithelial cells and BAL cells. Southern blot hybridization with an Alu gene sequence specific plasmid-pBLUR8 (kindly provided by Dr. R. Crystal, Cornell Medical School, New York, NY) was performed using the methodology described by Trapnell and colleagues (21). The buffer for reverse transcription of RNA consisted of 10 mM TRIS-HCl (pH, 9.3), 50 mM KCl, 3 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin (BSA), 0.5 mM

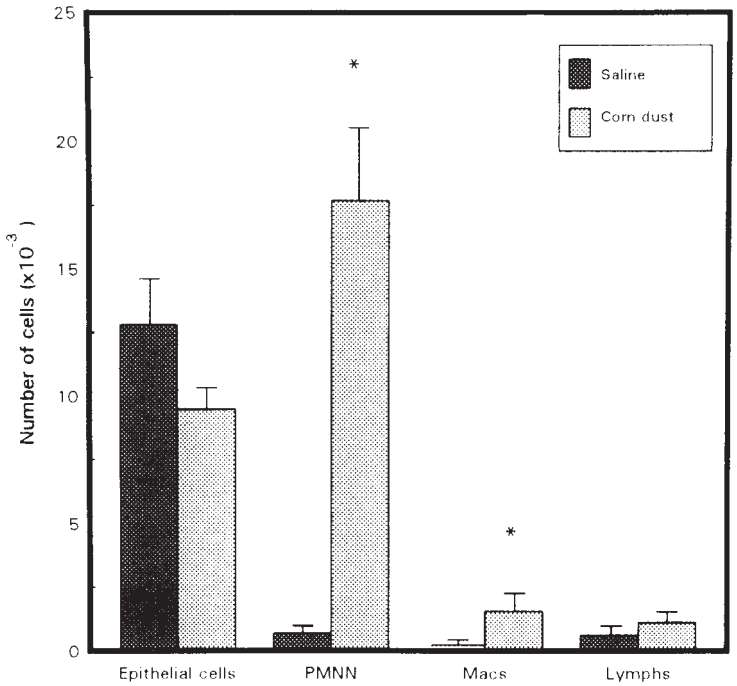

Figure 2. Inflammation in the endobronchial lavage fluid after inhalation of corn dust extract. Cell differentials in the endobronchial lavage fluid were evaluated on cytocentrifuge preparations and total number of the different cell types were calculated from the total cell count recovered. There was a significant increase in the PMN recovered in the airways lavage fluid ($p = 0.005$) as well as a small increase in macrophages ($p = 0.04$) after inhalation of corn dust extract.

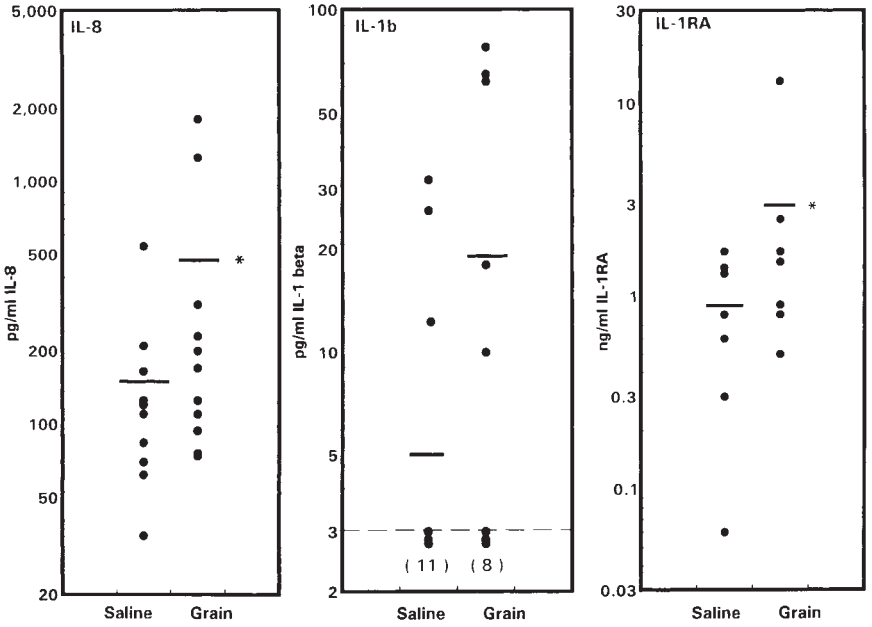

Figure 3. Cytokine levels in endobronchial lavage fluid after exposure to grain dust. IL-8, IL-1β, IL-1RA, IL-6, and TNF-α levels were determined in EBL 6 h after exposure to saline and grain dust extract. A significant increase in IL-8 ($p = 0.02$) and IL-1RA ($p = 0.03$) was found after exposure to the extract. The numbers in parentheses represent samples falling below the detection limit.

spermidine (all preceding chemicals from Sigma), 10 U/μl of M-MLV reverse transcriptase (RT) (BRL, Gaithersburg, MD), 0.5 mM dNTP (Pharmacia, Pleasant Hill, CA), 1 U/μl RNAsin (Promega, Madison, WI), and 5 mM random hexamers (Pharmacia). For polymerase chain reaction (PCR) amplification, 2 μl of cDNA were added to 48 μl PCR mix consisting of 10 mM TRIS-HCl buffer (pH, 9.3), 50 mM KCl, 3 mM $MgCl_2$, 0.1 mg/ml BSA, with 0.05 mM dNTP and 0.025 U/μl of Taq polymerase (Amplitaq; Cetus Corporation, Emeryville, CA). Sense and antisense primers for the different cytokines were present at 0.1 to 0.22 pM/μl. The primer pairs for IL-8 (22) were sense: TCTGCAGCTCTGTGTGAAGGTGCAGTT, antisense: AACCCTCTGCACCCAGTTTTCCTT; for TNF-α (23) were sense: CAGAGGGAAGAGTTCCCCAG, antisense: CCTTGGTCTGGTAGGAGACG; for IL-1β (23) were sense: AAACAGATGAAGTGCTCCTTCCAGG, antisense: TGGAGAACACCACTTGTTGCTCCA; for IL-6 (24) were sense: CCTTCTCCACAAGCGCCTTC, antisense: GGCAAGTCTCCTCATTGAATC; for sIL-1RA (25) were sense: GAATGGAAATCTGCAGAGGCCTCCGC, antisense: GGCACATCTTCCCTCCATGGATTCC. The PCR product generated with β-actin specific primers was used to verify similar cDNA input into each reaction well (8). The PCR products generated with each primer pair hybridized in a Southern blot with an antisense oligonucleotide specific for an internal sequence of each cytokine cDNA. For approximation of the number of cDNA molecules on a per cell basis, standard curves were generated from known amounts of plasmid containing the cDNA of interest. However, the difficulty in determining the precise concentration of plasmid by optical density (OD) 260/280 rendered cDNA copy number approximate rather than absolute. Therefore, cDNA corresponding to approximately 1 fg plasmid was expressed as 1 cDNA equivalent. The plasmid standard curves at 35 cycles of amplification were also used to set the detection limit for each chemokine cDNA. A product not visible (*see detection method below*) at this number of cycles indicated that the assay well contained < 100 specific cDNA molecules/well, corresponding to < 0.2 cDNA molecules generated by RT from the mRNA in one cell.

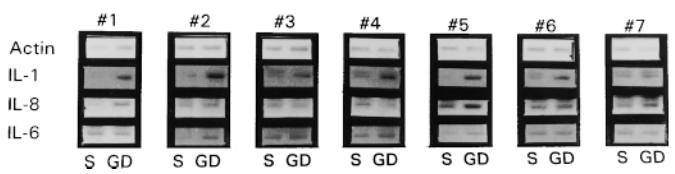

Figure 4. Representative RT/PCR products of amplified cytokine mRNA in seven individuals exposed to grain dust. PCR products of IL-1β, IL-8, and IL-6 mRNA from seven paired (S) and grain dust (GD)-exposed epithelial cell samples were electrophoresed onto agarose gels that were stained with ethidium bromide and photographed for densitometry quantitation of band optical density.

The PCR was performed in a 96-well thermocycler (MJ Research, Watertown, MA), 1 min at 94° C, 1.5 min at 56° C, and 2 min at 72° C. Ten microliters of the PCR product were run out on a 2% agarose gel (IBI, New Haven, CT) at 26, 29, and 31/32 cycles (35 cycles if no band was visible at 32 cycles) to ensure cycle-dependent increase in reaction product, as well as a linear relationship between input cDNA and PCR product in the standard curve. The gels were stained for 15 min with 5 μg/ml ethidium bromide and the DNA was then visualized on an ultraviolet (UV) illuminator and photographed with type 55 positive/negative film (Polaroid, Cambridge, MA). The negatives were scanned with a computerized laser densitometer (Bioimage; Millipore, Ann Arbor, MI).

Measurements of Cytokines in Endobronchial and Bronchoalveolar Lavage Fluids

IL-1β, IL-6, IL-8, and IL-1RA levels were determined by commercial sensitive and specific enzyme-linked immunosorbent assay (ELISA)s purchased from R&D Systems (Minneapolis, MN). TNF-α was determined using the TNF sensitive L929 mouse fibroblast cytotoxicity assay (26).

Immunocytochemistry

For immunostaining of cytokines in AM and PMN, 3 to 4×10^4 cells were centrifuged onto slides, which were air-dried for 2 h and then fixed with acetone for 10 min at room temperature. Then the slides were immersed for 20 min in phosphate-buffered saline (PBS) with 10% normal goat serum (PBS-GS), whereafter normal rabbit serum, rabbit antihuman IL-1β, or rabbit antihuman IL-8 antibody (Genzyme,

Cambridge, MA) at 1:500 dilution in PBS-GS was added for 2 h at room temperature. The slides were washed three times during 30 min with PBS, and biotinylated goat antirabbit 1 g (1:500 in PBS-GS) (Organon Technica, West Chester, PA) was added for 1 h. After rinsing the slides three times with PBS, alkaline-phosphatase-conjugated avidine, 1:300 dilution (Organon Technica), was added for 30 min, again followed by rinsing with PBS, as above. All slides were treated with Levamisol to inactivate any endogenous alkaline phosphatase and antibody-bound enzyme activity was then visualized by Naphtol AS-TR phosphate and Fast Red indicator dye (both from Sigma). IL-1RA was detected in cells using a goat antibody from R&D and an antigoat ABC kit according to manufacturer's directions (Vector Laboratories, Burlingame, CA). The enzyme reaction on all sides was terminated when the control slides started to show a weak red coloring.

Statistics

The distribution of the data required that nonparametric tests be performed, and the crossover design of the study allowed paired analyses. Thus, Wilcoxon's signed-rank test was used to make comparisons between the data generated from each inhalation challenge (27).

RESULTS

The mean age of the study subjects was 34.7 yr (range, 18 to 56 yr). The study subjects had been employed a mean of 10.1 yr (range, 1 to 25 yr) in the grain-handling industry. Eight of the subjects worked exclusively with corn, and seven worked at sites that processed mixed grains. All subjects were never smokers, and, as stipulated by our selection criteria, all subjects were nonatopic and had a negative airway response to inhaled histamine.

Inhalation challenge with buffered saline resulted in minimal increases in the FEV_1/FVC ratio (Figure 1). However, within 30 min after the inhalation challenge with corn dust extract, statistically significant, clinically relevant declines in FEV_1/FVC were observed. The obstructive physiology associated with the inhalation of corn dust extract persisted for the subsequent 5 h until bronchoscopy was performed.

Characterization of Cell Types Present in Endobronchial Lavage Fluid of Grain-Dust-exposed Subjects

The recovery of endobronchial lavage fluid was highly variable between subjects, with mean ± SD of 4.3 ± 2.7 ml recovered from subjects inhaling saline, and 3.3 ± 2.4 ml from subjects inhaling corn dust extract. The total number of cells recovered by the lavage was $1.3 \pm 1.5 \times 10^4$ from the saline-exposed subjects and $3.0 \pm 2.5 \times 10^4$ from the grain-dust-exposed subjects. The cell recovery was significantly higher after inhalation of corn dust extract ($p = 0.04$). The total number of the different cell types recovered in EBLs is shown in Figure 2. The number of PMNs in the EBL increased > 20-fold ($p = 0.005$), whereas epithelial cell and lymphocyte numbers were unchanged. The macrophage recovery was also increased after inhalation of grain dust ($p = 0.04$). No eosinophils (< 0.2%) were found in the endobronchial lavage fluid.

Cytokine Levels in Endobronchial Lavage Fluid after Exposure to Endotoxin-contaminated Grain Dust

Cytokine levels per milliliter endobronchial lavage fluid were measured by ELISA (Figure 3). IL-8 was present in EBL fluid

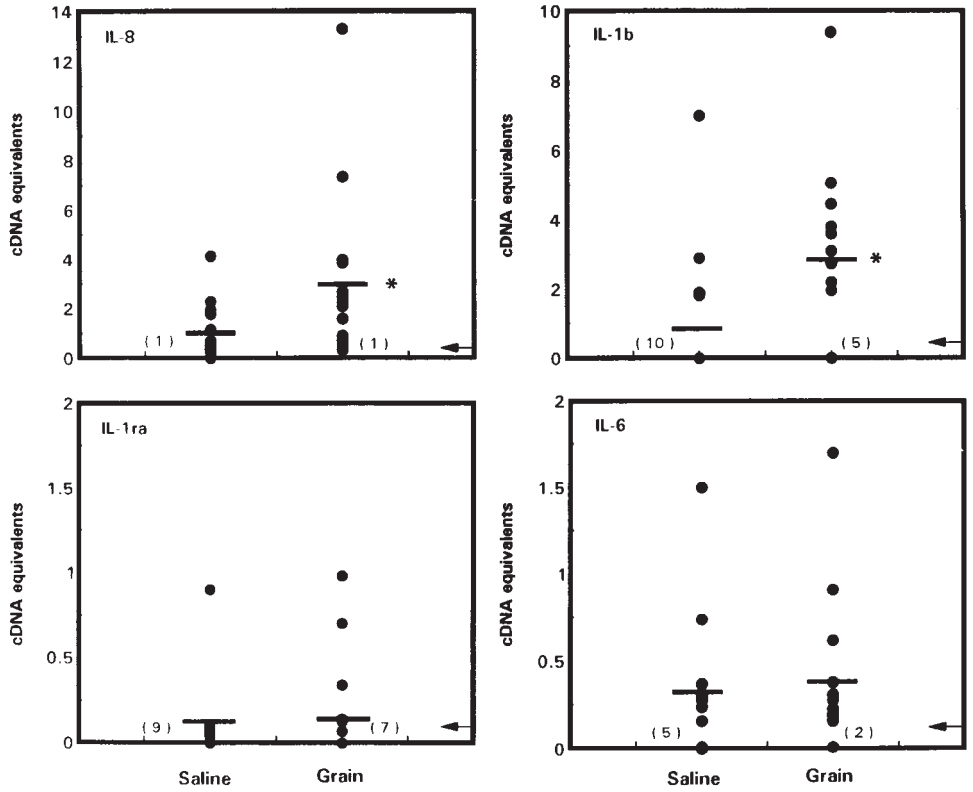

Figure 5. Cytokine mRNA levels in bronchial epithelium after exposure to grain dust. cDNA reverse-transcribed from RNA corresponding to approximately 400 cells was PCR amplified and then quantified for IL-8, IL-1β, IL-1RA, and IL-6 expression using cytokine specific plasmid standard curves amplified in parallel. A significant increase in IL-8 ($p = 0.02$) and IL-1β ($p = 0.03$) was found, whereas IL-6 and sIL-1RA mRNA levels were not affected. The numbers in parentheses represent samples falling below the detection limit.

after inhalation of either saline or corn dust extract, but the grain dust samples showed 3.2-fold increased concentrations ($p = 0.02$). IL-1β was not significantly different after inhalation of saline versus corn dust extract. However, high levels of IL-1RA were detected in all fluids assayed, with a significant 3-fold increase after grain dust exposure ($p = 0.03$). Neither IL-6 nor TNF-α were found in the endobronchial lavage fluid.

Cytokine mRNA Expression in Airway Epithelium after Exposure to Grain Dust

Bronchial cells, obtained by endobronchial brush biopsy, were disaggregated, and differential counts were performed on cytocentrifuge preparations. The preparations from corn-dust-exposed subjects contained a small but significantly increased proportion of PMNs; a mean ± SD of 4.4 ± 1.0% in the grain-dust-exposed population as compared with 1.4 ± 0.3% in the saline-exposed population. The remaining cells were epithelial cells, with approximately 50% of the cells having ciliated cell morphology. The PMNs were not separated from the epithelial cells before RNA preparation. RNA corresponding to 10^4 cells was reverse-transcribed, and relative cDNA levels for IL-1β, sIL-1RA, TNF-α, IL-6, and IL-8 from cells obtained after exposure to saline and corn dust were determined by semiquantitative PCR using either cDNA from lipopolysaccharide (LPS)-stimulated macrophages or plasmid preparations containing cytokine cDNA as dose-response curves. Representative cytokine PCR products from seven paired samples are shown in Figure 4. In Figure 5, the quantified cytokine data from all the epithelial cell specimens obtained after inhalation of either saline or corn dust extract are shown. A small but significant increase in IL-8 (3.0-fold, $p = 0.02$) and IL-1β (2.7-fold, $p = 0.03$) was found after exposure to grain dust, whereas IL-6 levels were unchanged. TNF-α levels were below our set limit for detection (< 0.2 cDNA molecules yield/cell by reverse transcription), although a weak product band was detected in the gels after 35 cycles of amplification. Of the samples analyzed for sIL-1RA expression, two of 11 of the saline-exposed BEs showed a positive signal and four of 11 corn-dust-exposed BEs had sIL-1RA mRNA levels above the detection limit.

Comparison of Cytokine mRNA Levels in Airway Epithelium and in BAL Cells after Inhalation of Corn Dust Extract

To relate the mRNA levels in airway epithial cells from subjects inhaling corn dust extract to mRNA levels in BAL cells (from the same subjects), RNA from the same number of cells of each cell population was reverse-transcribed and cDNA levels were determined by PCR. The data comparing cytokine mRNA levels in 14 BE and BAL cell samples are summarized in Figure 6. BAL cells exposed to corn dust extract expressed 11-fold more IL-1β mRNA than did epithelial cells ($p < 0.001$) and 2.5 times the amount of IL-6 ($p = 0.06$), whereas epithelial cells expressed slightly more IL-8 mRNA ($p > 0.05$). sIL-1RA and TNF-α were present at very high levels in the BAL cells compared with expression in BE cells ($p < 0.001$).

Immunocytochemical Detection of Cytokines and sIL-1RA in Alveolar Macrophages and Inflammatory Granulocytes

BAL cells obtained after exposure to grain dust contained approximately 70% PMNs. Because these granuloctyes have been shown to have the ability to produce all the cytokines of interest in this study, protein expression of these cytokines by PMN and AM was compared by immunocytochemical staining of the cells. It can be seen in Figure 7 that AMs from grain-dust-exposed subjects are positive for IL-1β, IL-6, and IL-8

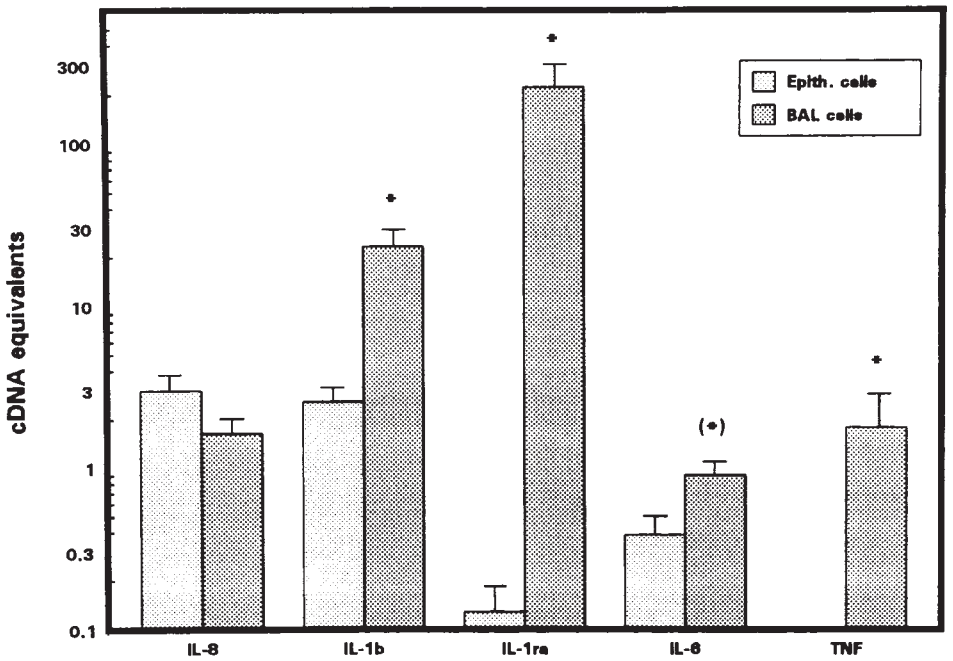

Figure 6. Comparison of cytokine mRNA expression in bronchial epithelium and in cells obtained by bronchoalveolar lavage after exposure to grain dust. RNA from the same number of bronchial cells and BAL cells was reverse-transcribed and then amplified by PCR for cytokine mRNA estimation. A similar amount of IL-8 mRNA was expressed in BE and BAL cells. IL-1β, IL-1RA were significantly higher in the BAL cells (*$p < 0.001$), whereas IL-6 was marginally higher in the BAL cells (*$p = 0.06$).

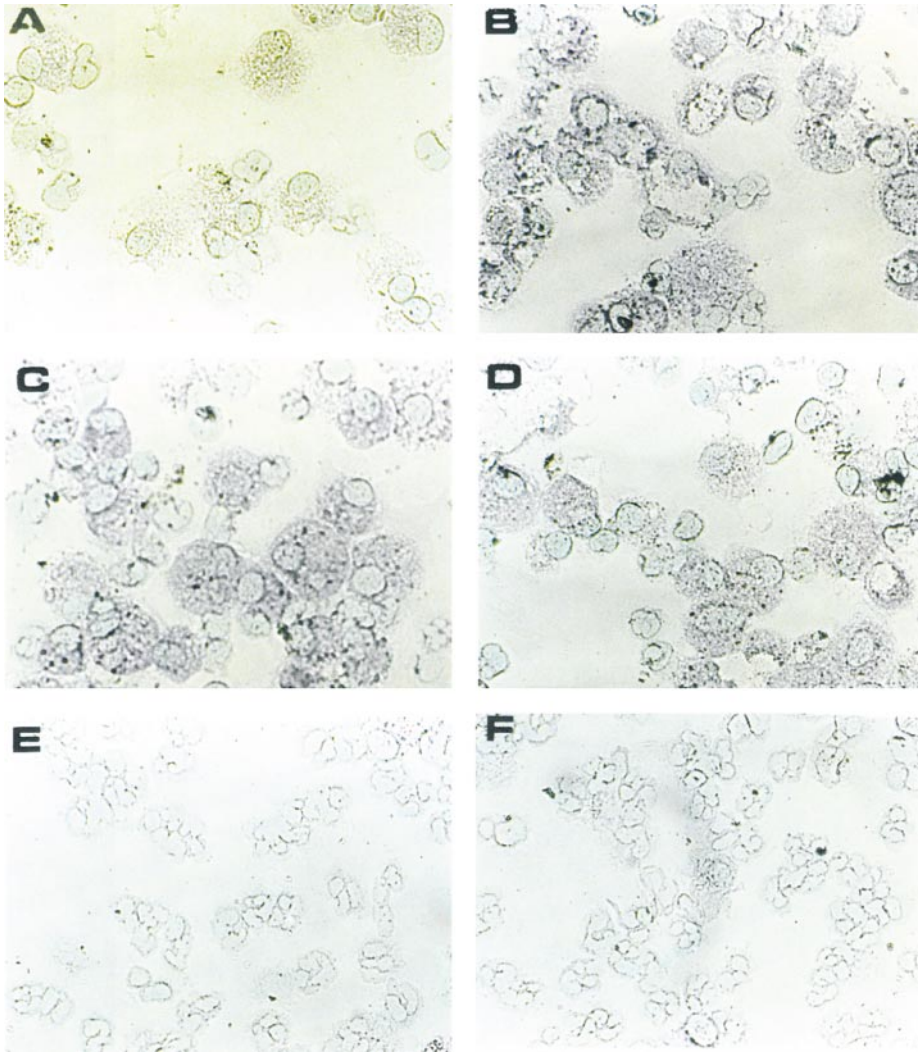

Figure 7. Immunochemical localization of cytokines in alveolar macrophages of grain-dust-exposed subjects. Cytocentrifuge preparations of BAL cells separated into AM (*panels A–D*) and PMN (*panels E* and *F*) by density gradient were treated with control antibody (*panel A*), antibody to IL-1β (*panels B* and *E*), antibody to IL-6 (*panel C*), and antibody to IL-8 (*panels D* and *F*). A variable proportion of AM were positive for the cytokines, whereas PMN were completely negative under the same staining conditions.

proteins, whereas the inflammatory PMNs are negative for these cytokines under the identical staining conditions. BAL cells were stained for IL-1RA protein expression. Strong immunoreactivity was found in both AM and PMN (Figure 8B) relative to the cells reacted with control antibody (Figure 8A).

Comparison of Cytokine Levels in Endobronchial and Bronchoalveolar Lavage Fluid

To evaluate if the differences in mRNA expression between cells from the bronchial region and the alveolar region were reflected in cytokine protein levels in the EBL and BAL fluids, the cytokine profile in EBL was compared with that of BAL. Because the lining fluid dilution certainly is different in the lavage fluids from the two locations, the cytokine data are expressed as a BAL:EBL ratio. That IL-8 is concentrated in central airways can be seen in Figure 9, consistent with the finding that epithelial cells preferentially produce more IL-8 mRNA than alveolar cells. Because IL-6 and TNF-α were below the detection limit of 3 pg/ml in the EBL, ratios for these cytokines exceeded 100 and 10.

DISCUSSION

The results from this investigation demonstrate that the inflammatory response to inhaled grain dust appears to be compartmentalized. Although IL-8 is primarily produced by airway epithelial cells and inflammatory cells surrounding the airway, other proinflammatory cytokines such as IL-1β, IL-6, and TNF-α are primarily produced by alveolar macrophages. These results suggest that the local production and release of IL-8 in and around the airway serves to localize the inflammatory response to the airway, which ultimately may lead to airflow obstruction.

Macrophages and neutrophils appear to play pivotal roles in the initial inflammatory response to inhaled grain dust. *In vitro*, grain dust is directly chemotactic for neutrophils (28), and can induce alveolar macrophages to release IL-1 (29) and other mediators that have potent chemotactic activity for neutrophils (28). Inhalation studies in humans (3, 30, 31) and in mice (31) have shown that after a single exposure to grain dust, neutrophils are rapidly recruited to the lung and proinflammatory cytokines (IL-1β, TNF-α, and IL-6) and chemo-

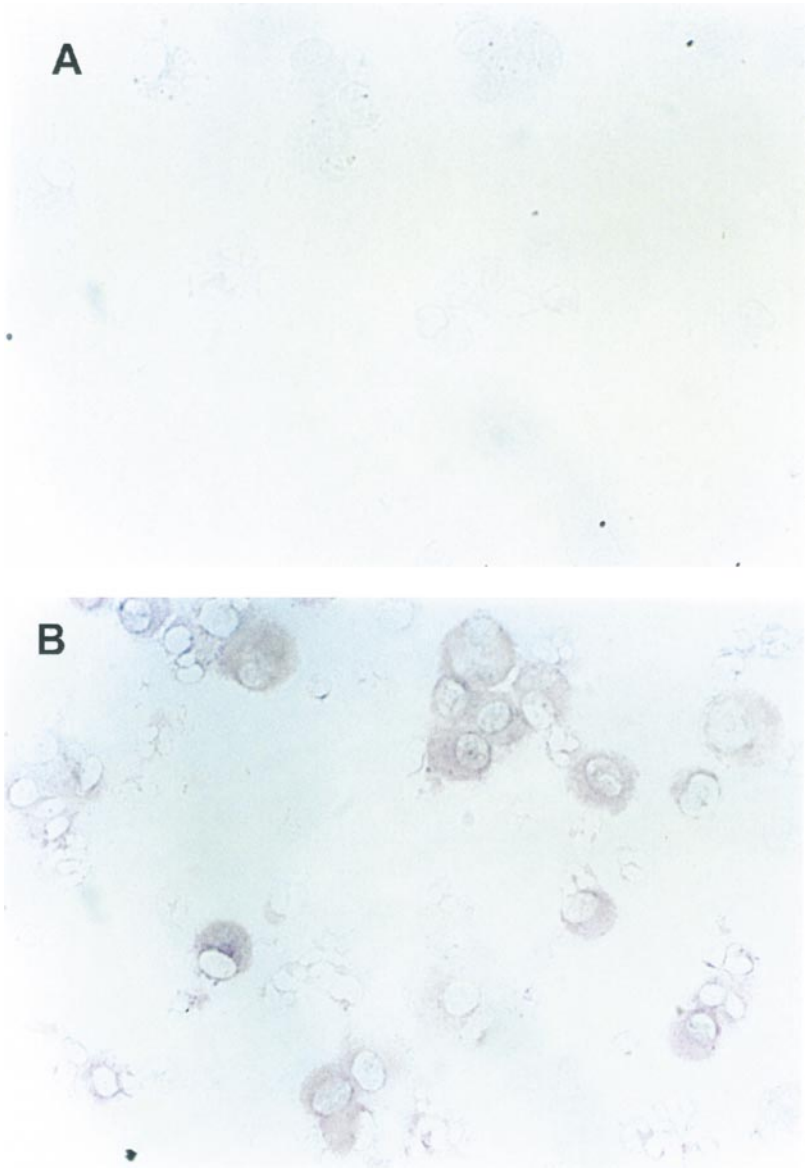

Figure 8. Immunocytochemical localization of IL-1RA in alveolar macrophages and inflammatory granulocytes 6 h after inhalation of corn dust extract. Cytocentrifuge preparations of BAL cells (65% AM, 32% PMN) obtained from subjects after inhalation of corn dust extract were stained for IL-1RA. Both AM and PMN were strongly positive for IL-1RA (*panel B*) as compared with cells reacted with control antibody (*panel A*).

kines (IL-8 and macrophage inflammatory protein [MIP]-2) are produced and released for as long as 48 h (31). Immunohistochemical staining and *in situ* hybridization (32) indicate that the macrophage and the neutrophil are actively involved in the de novo synthesis of these proinflammatory agents. In rats challenged with aerosolized LPS, the influx of neutrophils and bronchial hyperreactivity was inhibited by prior treatment with TNF-α specific antibodies (33), suggesting that the proinflammatory cytokines are essential to the recruitment of PMNs.

Epithelial cells may also be involved in recruitment and modulation of the inflammatory response to inhaled grain dust. Epithelial cells (A549 and bronchial epithelia) require a specific host-derived signal (TNF-α or IL-1) for induction of IL-8 (15). In a baboon model of sepsis, pretreatment with anti-TNF-α antibody significantly reduced the circulating concentration of IL-8 (34), suggesting that TNF-α and/or IL-1 are needed to stimulate other cells to release IL-8 and promote neutrophil chemotaxis. MIP-2 is thought to be the murine homologue of IL-8 (15), has potent chemotactic activity for neutrophils (35), is a member of the IL-8 supergene family (15), and has been shown to be upregulated in rat lungs after intraperitoneal endotoxin challenge (36). In this study, we report that human IL-8 is produced and released by airway epithelia after an inhalation challenge with grain dust, suggesting that airway epithelia are activated either directly by grain dust or by cells or cell products that come in contact with the apical or basolateral portion of these cells. In aggregate, these findings suggest that inhaled grain dust initiates a complex interaction between inflammatory (primarily macrophages and neutrophils) and structural (airway epithelial) cells, and this interaction is mediated by specific proinflammatory cytokines and

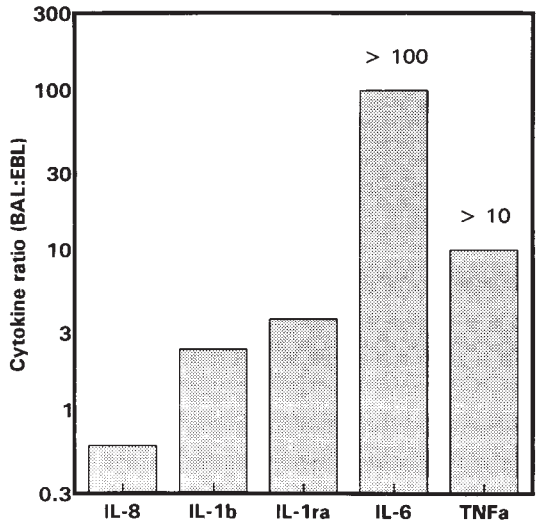

Figure 9. Comparison of cytokine levels in endobronchial and bronchoalveolar lavage fluids after exposure to corn dust extract. EBL and BAL fluids were tested for cytokines by ELISA and bioassay (TNF-α). Levels of the different cytokines in the two sites were compared as a ratio to indicate the preferential site of cytokine production.

chemokines that are produced and released in the airway lumen and possibly the interstitium of the lung.

IL-1RA appears to be released as a homeostatic mechanism to limit the inflammatory response. High amounts of IL-1RA have been previously found in normal BAL fluid, and AMs were found to constitutively contain IL-1RA protein (37). In this study, we have shown that whereas sIL-1RA was found in low concentration in airway epithelial cells, it was present at a high concentration in saline-exposed EBL and was significantly increased after inhalation of corn dust extract. In the rat, high levels of IL-1RA mRNA were found in both AM and PMN isolated 6 h after intratracheal administration of endotoxin (38). Because the bronchial lining fluid contains macrophages and increased numbers of PMNs after grain dust exposure, it is possible that elevated levels of IL-1RA/ml EBL fluid originate from both the stimulated AMs and the PMNs. Indeed, both PMNs and AMs in the BAL fluid from grain-dust-exposed subjects contained IL-1RA protein, although these PMNs did not show cytokine immunoreactivity. Interestingly, *in vitro* studies have shown that AMs have a poor sIL-1RA response to LPS. Culture alone, in the absence of adherence, increases sIL-1RA mRNA levels and protein secretion, and the effect of LPS is minimal compared with the response to IL-4 (37, 39). IL-4 is unlikely to be involved in the acute response to grain dust or endotoxin, suggesting that other not yet identified inflammatory mediators stimulate gene expression *in vivo*. sIL-1RA has been shown to inhibit a number of IL-1α- and IL-1β-dependent functions such as thymocyte proliferation, synthesis of prostaglandin E_2, and collagenase production by fibroblasts through competition for IL-1 receptor binding on the target cells (40). The ratio of IL-1RA to IL-1β in the EBL was more than 100:1 in most saline and corn-dust-exposed fluids, suggesting that the proinflammatory effects of IL-1β after inhaling corn dust may be controlled by the secretion of sIL-1RA.

In conclusion, the acute inflammatory response 6 h post-exposure to corn dust extract clearly involves the airway epithelium in the production of IL-8 and possibly IL-1β. Because elevated IL-6 and TNF-α mRNA levels were found only in the BAL cells and proteins were detected only in the BAL fluid, it is likely that the bronchoalveolar region is the primary site for production of these cytokines. Among the BAL cells, AMs and not PMNs appeared to be the major producers of IL-1β, IL-6, and IL-8 as determined by immunohistochemistry, whereas IL-1RA was produced by both AMs and PMNs. These results clearly indicate that the inflammatory response to inhaled grain dust is compartmentalized, involving macrophages, neutrophils, and airway epithelial cells.

References

1. Huy, T., K. de Schipper, M. Chan-Yeung, and S. M. Kennedy. 1991. Grain dust and lung function: dose-response relationships. *Am. Rev. Respir. Dis.* 144:1314–1321.
2. von Essen, S. G., A. B. Thompson, R. A. Robbins, K. K. Jones, C. A. Dobry, and S. I. Rennard. 1990. Lower respiratory tract inflammation in grain farmers. *Am. J. Ind. Med.* 17:75–76.
3. Clapp, W. D., S. Becker, J. Quay, J. L. Watt, P. S. Thorne, K. L. Frees, X. Zhang, C. R. Lux, and D. A. Schwartz. 1994. Grain dust-induced airflow obstruction and inflammation of the lower respiratory tract. *Am. J. Respir. Crit. Care Med.* 150:611–617.
4. Schwartz, D., P. Thorne, P. Jagielo, G. White, S. Bleuer, and K. Frees. 1994. Endotoxin responsiveness and grain dust-induced inflammation in the lower respiratory tract. *Am. J. Physiol.* 267:L609–L617.
5. Castellan, R. M., S. A. Olenchock, K. B. Kinsely, and J. L. Hankinson. 1987. Inhaled endotoxin and decreased spirometric values. *N. Engl. J. Med.* 317:605–610.
6. Gordon, T., J. Balmes, J. Fine, and D. Sheppard. 1991. Airway oedema and obstruction in guinea pigs exposed to inhaled endotoxin. *Br. J. Ind. Med.* 48:629–635.
7. Becker, S., H. S. Koren, and D. C. Henke. 1993. Interleukin-8 expression in normal nasal epithelium and its modulation by infection with respiratory syncytial virus and cytokines tumor necrosis factor, interleukin-1, and interleukin-6. *Am. J. Respir. Cell Mol. Biol.* 8:20–27.
8. Becker, S., J. Quay, H. S. Koren, and J. S. Haskill. 1994. Constitutive and stimulated MCP-1, GRO alpha, beta, and gamma expression in human airway epithelium and bronchoalveolar macrophages. *Am. J. Physiol.* 266:L278–L286.
9. Mattoli, S., F. Colotta, G. Fincato, M. Mezzetti, A. Mantovani, F. Patalano, and A. Fasoli. 1991. Time course of IL1 and IL6 synthesis and release in human bronchial epithelial cell cultures exposed to toluene diisocyanate. *J. Cell. Physiol.* 149:260–268.
10. Standiford, T. J., S. L. Kunkel, M. A. Basha, S. W. Chensue, J. P. Lynch, G. B. Toews, J. Westwick, and R. M. Strieter. 1990. Interleukin-8 gene expression by a pulmonary epithelial cell line: a model for cytokine networks in the lung. *J. Clin. Invest.* 86:1945–1953.
11. Noah, T. L., and S. Becker. 1993. Respiratory syncytial virus-induced cytokine production by a human bronchial epithelial cell line. *Am. J. Physiol.* 265:L472–L478.
12. Devlin, R. B., T. L. McKinnon, T. L. Noah, S. Becker, and H. S. Koren. 1994. Cytokine and fibronectin production by human alveolar macrophages and airway epithelial cells exposed to ozone in vitro. *Am. J. Physiol.* 266:L612–L619.
13. Marini, M., E. Vittori, J. Hollemborg, and S. Mattoli. 1992. Expression of the potent inflammatory cytokines, granulocyte-macrophage-colony-stimulating factor and interleukin-6 and interleukin-8, in bronchial epithelial cells of patients with asthma. *J. Allergy Clin. Immunol.* 89:1001–1009.
14. Ohnishi, T., C. Vancheri, G. Cox, J. Gauldie, J. Dolovich, J. A. Denburg, and M. Jordana. 1991. Monocyte-macrophage differentiation induced by human upper airway epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 4:255–263.
15. Strieter, R. M. 1993. Interleukin-8. *In* J. Kelley, editor. Cytokines of the Lung. Marcel Dekker, New York. 281–305.
16. American Thoracic Society. 1991. Lung function testing: selection of reference values and interpretative strategies. *Am. Rev. Respir. Dis.* 144: 1202–1218.
17. Schwartz, D. A., P. S. Thorne, S. J. Yagla, L. F. Burmeister, S. A. Olenchock, J. L. Watt, and T. J. Quinn. 1995. The role of endotoxin in grain dust-induced lung disease. *Am. J. Respir. Crit. Care Med.* 152:603–608.
18. American Thoracic Society. 1985. Summary and recommendations of a workshop on the investigative use of bronchoscopy and bronchoalveolar lavage in asthmatics. *Am. Rev. Respir. Dir.* 132:180–182.

19. Eschenbacher, W. L., and T. R. Gravelyn. 1987. A technique for isolated airway segment lavage. *Chest* 92:105–109.
20. Brenner, C. A., A. W. Tam, P. A. Nelson, E. G. Engleman, N. Suzuki, K. E. Fry, and J. W. Larrick. 1989. Message amplification phenotyping (MAPPing): a technique to simultaneously measure multiple mRNAs from small numbers of cells. *Biotechniques* 7:1096–1103.
21. Trapnell, B. C., C. S. Chu, P. K. Paakko, T. C. Banks, K. Yoshimura, V. J. Ferrans, M. S. Chernick, and R. G. Crystal. 1991. Expression of the cystic fibrosis transmembrane conductance regulator gene in the respiratory tract of normal individuals and individuals with cystic fibrosis. *Proc. Natl. Acad. Sci. U.S.A.* 88:6565–6569.
22. Matsushima, K., K. Morishita, T. Yoshimura, S. Lavu, Y. Kobayashi, W. Lew, E. Appella, H. F. Kung, E. J. Leonard, and J. J. Oppenheim. 1988. Molecular cloning of a human monocyte-derived neutrophil chemotactic factor (MDNCF) mRNA by interleukin-1 and tumor necrosis factor. *J. Exp. Med.* 167:1883–1893.
23. Wang, A. M., M. V. Doyle, and D. F. Mark. 1989. Quantitation of mRNA by the polymerase chain reaction. *Proc. Natl. Acad. Sci. U.S.A.* 86:9717.
24. Hirano, T., K. Yasukawa, H. Harada, T. Taga, Y. Watanabe, T. Matsuda, S. Kashiwamura, K. Nakajima, K. Koyama, and A. Iwamatsu. 1986. Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. *Nature* 324: 73–76.
25. Haskill, S., G. Martin, L. Van Le, J. Morris, A. Peace, C. F. Bigler, G. J. Jaffe, C. Hammerberg, S. A. Sporn, and S. Fong. 1991. cDNA cloning of an intracellular form of the human interleukin 1 receptor antagonist associated with epithelium. *Proc. Natl. Acad. Sci. U.S.A.* 88:3681–3685.
26. Leeper-Woodford, S. K., P. D. Carey, K. Byrne, J. K. Jenkins, B. J. Fisher, C. Blocher, H. J. Sugerman, and A. A. Fowler. 1991. Tumor necrosis factor: alpha and beta subunits appear in the circulaton during onset of sepsis-induced lung injury. *Am. Rev. Respir. Dis.* 143:1076–1082.
27. Rosner, B. 1990. Fundamentals of Biostatistics, 3rd ed. PWS-Kent Publishing Company, Boston, MA.
28. Von Essen, S. G., R. A. Robbins, A. B. Thompson, R. F. Ertl, J. Linder, and S. Rennard. 1988. Mechanisms of neutrophil recruitment to the lung by grain dust exposure. *Am. Rev. Respir. Dis.* 138:921–927.
29. Lewis, D. M., and M. S. Mentnech. 1984. Extracts of airborne grain dusts simulate interleukin-1 (IL-1) production by alveolar macrophages (abstract). *Am. Rev. Respir. Dis.* 129:A161.
30. Von Essen, S. G., S. McGranaghan, D. Cirian, D. O'Neill, J. R. Spurzem, and S. I. Rennard. 1991. Inhalation of grain sorghum dust extract causes respiratory tract inflammation in human volunteers (abstract). *Am. Rev. Respir. Dis.* 143:A105.
31. Deetz, D. C., P. J. Jagielo, T. J. Quinn, P. S. Thorne, S. A. Bleuer, and D. A. Schwartz. 1997. The kinetics of grain dust-induced inflammation of the lower respiratory tract. *Am. J. Respir. Crit. Care Med.* 155: 254–259.
32. Wohlford-Lenane, C. L., D. C. Deetz, and D. A. Schwartz. 1998. Cytokine gene expression following inhalation of grain dust and lipopolysaccharide. *Am. J. Physiol.* (In press)
33. Kips, J. C., J. Tavernier, and R. A. Pauwels. 1992. Tumor necrosis factor causes bronchial hyperresponsiveness in rats. *Am. Rev. Respir. Dis.* 145: 332–336.
34. Redl, H., G. Schlag, M. Ceska, J. Davies, and W. A. Buurman. 1993. Interleukin-8 release in baboon septicemia is partially dependent on tumor necrosis factor. *J. Infect. Dis.* 167:1464–1466.
35. Wolpe, S. D., B. Sherry, D. Juers, G. Davatelis, R. W. Yurt, and A. Cerami. 1989. Identification and characterization of macrophage inflammatory protein 2. *Proc. Natl. Acad. Sci. U.S.A.* 86:612–616.
36. Rose, C. E., C. A. Juliano, D. E. Tracey, T. Yoshimura, and S. M. Fu. 1994. Role of interleukin-1 in endotoxin-induced lung injury in the rat. *Am. J. Respir. Cell Mol. Biol.* 10:214–221.
37. Moore, S. A., R. M. Streiter, M. W. Rolfe, T. J. Standiford, M. D. Burdick, and S. L. Kunkel. 1992. Expression and regulation of human alveolar macrophage-derived interleukin-1 receptor antagonist. *Am. J. Respir. Cell Mol. Biol.* 6:569–575.
38. Ulich, T. R., K. Guo, S. Yin, J. del Castillo, E. S. Yi, R. C. Thompson, and S. P. Eisenberg. 1992. Endotoxin-induced cytokine gene expression *in vivo*: IV. Expression of interleukin-1α/β and interleukin-1 receptor antagonist mRNA during endotoxemia and during endotoxin-initiated local acute inflammation. *Am. J. Pathol.* 141:61–68.
39. Galve-de Rochemonteix, B., L. P. Nicod, R. Chicheportiche, S. Lacraz, C. Baumberger, and J. M. Dayer. 1993. Regulation of interleukin-1ra, interleukin-1 alpha, and interleukin-1 beta production by human alveolar macrophages with phorbol myristate acetate, lipopolysaccharide, and interleukin-4. *Am. J. Respir. Cell Mol. Biol.* 8:160–168.
40. Dinarello, C. A., and R. C. Thompson. 1991. Blocking IL-1: interleukin 1 receptor antagonist *in vivo* and *in vitro*. *Immunol. Today* 12:404–410.